

# Soil Column Evaluation of Factors Controlling Biodegradation of DNT in the Vadose Zone

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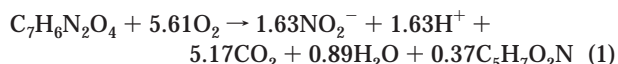
High concentrations of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are present in vadose zone soils at many facilities where explosives manufacturing has taken place. Both DNT isomers can be biodegraded under aerobic conditions, but rates of intrinsic biodegradation observed in vadose zone soils are not appreciable. Studies presented herein demonstrate that nutrient limitations control the onset of rapid 2,4-DNT biodegradation in such soils. In column studies conducted at field capacity, high levels of 2,4-DNT biodegradation were rapidly stimulated by the addition of a complete mineral medium but not by bicarbonate-buffered distilled deionized water or by phosphate-amended tap water. Biodegradation of 2,6-DNT was not observed under any conditions. Microcosm studies using a DNT-degrading culture from column effluent suggest that, after the onset of 2,4-DNT degradation, nitrite evolution will eventually control the extent of degradation achieved by two mechanisms. First, high levels of nitrite (40 mM) were found to strongly inhibit 2,4-DNT degradation. Second, nitrite production reduces the solution pH, and at pH levels below 6.0, 2,4-DNT degradation slows rapidly. Under conditions evaluated in laboratory-scale studies, 2,4-DNT biodegradation enhanced the rate of contaminant loss from the vadose zone by a factor of 10 when compared to the washout due to leaching.

## Introduction

Dinitrotoluenes (DNT) are formed in the second step of toluene nitration during 2,4,6-trinitrotoluene (TNT) synthesis. In particular, two DNT isomers [2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT)] are formed at yields of 76% and 19%, respectively (a small percentage of other isomers are produced) (1). Poor handling and disposal practices associated with the production of TNT have led to substantial DNT contamination problems at ammunition production and handling facilities worldwide (2). 2,4-DNT and 2,6-DNT are both listed as priority pollutants by the U.S. EPA, thus requiring remediation at contaminated sites.

Because both DNT isomers can be biodegraded by aerobic bacteria, bioremediation of contaminated media has been an area of research and development for some time. The use of various ex-situ processes to biodegrade DNT in soil and groundwater has been reported (3–9), but little attention has been given to in-situ processes (either natural attenuation or accelerated bioremediation) despite the ability of specific bacteria to use both 2,4-DNT and 2,6-DNT as carbon, energy, and nitrogen sources (3, 10–15).

During aerobic biodegradation, DNT depletion and oxygen consumption are coupled with nitrite evolution, CO<sub>2</sub> production, and biomass growth (10–12). Daprato et al. (16) described the stoichiometry of the overall process (eq 1) using a simple empirical formula for biomass composition (C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N):



On the basis of this stoichiometry and observations in laboratory studies, it would seem as if oxygen and appropriate microbes would be sufficient for the onset of DNT degradation. Both isomers, however, often display an unexpectedly high degree of recalcitrance in aerobic environments. This is particularly true in contaminated vadose zone soils where much of the existing DNT contamination resides.

The observed persistence of DNT isomers in vadose zone soils raises questions regarding the factor(s) that limit natural biodegradation processes, the extent to which natural bioattenuation may be lowering contaminant levels, and the ability to induce activity in-situ. Whereas the availability of carbon or nitrogen is not a concern, several other factors may result in the recalcitrance nature of DNT in vadose zone soils. First is the potential absence of DNT-degrading strains; however, we have been able to culture DNT degraders from all historically contaminated sites examined to date (17). Second is the potential for high concentrations of DNT to be inhibitory or toxic to bacteria. Previous studies in ex-situ reactors demonstrated that high concentrations of 2,4-DNT will inhibit 2,6-DNT degradation but not 2,4-DNT degradation. Other possible factors that may contribute to the recalcitrance of DNT include demands for phosphorus (15, 16, 18) (or other essential nutrients), low pH from nitrite production during DNT biodegradation (4, 15, 18), and the accumulation of inhibitory nitrite levels when the replenishment of water does not allow for significant nitrite dilution (17).

In this paper, we present the results of a laboratory-scale evaluation of factors that control the aerobic biodegradation of DNT in a simulated vadose zone. Aged vadose zone soils containing a high concentration of 2,4-DNT and a low concentration of 2,6-DNT were obtained from Badger Army Ammunition Plant (BAAP) in Baraboo, WI. Initial experiments were conducted in column systems to assess the factors required to initiate rapid biodegradation of DNT. Additional column studies were conducted to investigate how the operational parameters of interest in vadose zone bioremediation, including aeration frequency and water recycling, will impact the degradation activity. A series of respirometer studies were conducted using cultures obtained from column systems to further investigate the factors that control the extent of degradation achievable before nitrite accumulation, pH drop, or nutrient limitations slow degradation activity. Results from these studies suggest that, at field capacity, nutrient availability is the controlling factor in the onset of

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TABLE 1. BAAP Soil Characteristics

parameter	value	method
<b>Physical Parameters</b>		
pH	7.4	
sat paste moisture (%)	17.7	
moisture (%)	0.4	
SP EC (mS/cm)	2.0	
texture (%)		3.4.3.5 <sup>a</sup>
sand	90.0	
silt	8.0	
clay	2.0	
soil class	sand	
bulk density	1.99 g/cm <sup>3</sup>	14–3 <sup>b</sup>
moisture retention (0.33 bar)	4.2%	
<b>Plant Available Nutrients (mg/kg)</b>		
ammonia-N	3.0	350.3 <sup>c</sup>
nitrate-N	4.0	353.2 <sup>c</sup>
nitrite-N	<1.0	353.2 <sup>c</sup>
o-phosphate	30.0	365.4 <sup>c</sup>
copper	2.1	200.7 <sup>c</sup>
iron	8.1	200.7 <sup>c</sup>
manganese	6.4	200.7 <sup>c</sup>
zinc	2.8	200.7 <sup>c</sup>
molybdenum	<0.05	200.7 <sup>c</sup>

<sup>a</sup> EPA 600/4-79-020. <sup>b</sup> Blake, G. R.; Hartage, K. H. In *Methods of Soil Analysis Part 1: Physical and Mineralogical Methods*. Klute, A., Ed.; American Society of Agronomy: Madison, WI, 1986; pp 363–375. <sup>c</sup> EPA 600/2-78-054.

activity and that the extent of degradation that follows will be controlled by nitrite accumulation.

## Materials and Methods

**Chemicals.** 2,4-Dinitrotoluene (97%) and 2,6-dinitrotoluene (98%) were obtained from Aldrich (Milwaukee, WI). The following reagent grade chemicals were used as media constituents: CaSO<sub>4</sub>·5H<sub>2</sub>O, MgSO<sub>4</sub>·7H<sub>2</sub>O, FeSO<sub>4</sub>·7H<sub>2</sub>O, CaCl<sub>2</sub>·7H<sub>2</sub>O, NaCl, ZnSO<sub>4</sub>·7H<sub>2</sub>O, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, and H<sub>3</sub>BO<sub>3</sub> (Fisher Scientific, J. T. Baker Chemical Co., and Mallinckrodt Inc.). All reagent grade buffers for media, KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, NaHCO<sub>3</sub>, and Na<sub>5</sub>P<sub>3</sub>O<sub>10</sub> were obtained from Fisher Scientific.

**Soil Preparation and Analysis.** Aged DNT-laden soil was collected previously from BAAP (Baraboo, WI) at an average boring depth of 30 ft and homogenized as described by Zhang et al. (15). Initial DNT concentrations in the soil were determined as described by Zhang et al. (15). Soil contained 8700 (± 420) mg of 2,4-DNT and 148 (± 14) mg of 2,6-DNT/kg of soil (dry basis). This composite soil was used for all soil column experiments. Additional soil analysis was performed by Soil Analytical Services Inc. (College Station, TX). The analytical methods used for soil characterization and corresponding results are reported in Table 1.

**Analytical Methods.** DNT analysis was performed on a Waters Millennium II HPLC system equipped with a diode array detector with compounds quantified at A<sub>230</sub>. Initially, separation was achieved at ambient temperature with a Nova-Pak C<sub>18</sub> 60 Å 4-μm silica-based HPLC column (3.9 × 150 mm, Waters, USA) with a mobile phase of 2-propanol/water (19:81) at 1 mL/min. Subsequently, HPLC was performed with a Hypercarb porous graphite column (5 μm × 150 mm, Thermohypersil, U.K.) with a mobile phase of acetonitrile/water (90:10) containing trifluoroacetic acid (0.55 mL/L) (3).

Nitrite analysis was performed using a colorimetric method, coupling diazotized sulfanilamide with *N*-(1-naphthyl)ethylenediamine producing an azo dye, and measured using a spectrophotometer (Turner SP-830; Dubuque, IA) at 543 nm (19). Soil nitrite extraction was performed with 2 M KCl solution displacing soil-bound NO<sub>2</sub><sup>-</sup> into solution (20). Nitrate analysis was performed with ion chromatography (Dionex IC20, Sunnyvale, CA) (21).

**Column Studies.** BAAP soil was placed into custom-fabricated plexiglass columns (10.2 cm i.d. × 30.5 cm) designed to support the soil above the column base to allow for sample collection and to provide an even flow of air into the bottom of the soil pack. To support the soil, wire mesh cloth (0.145 cm) was used and held in place above the bottom of the column (3.8 cm) by four small plexiglass rods (0.64 cm diameter). A layer of gravel (7.6 cm) was placed on the wire mesh to prevent soil washout. Soil (2 kg) was placed in the columns at even intervals (5 intervals, 400 g each) with consistent packing to minimize variation between columns. A layer of gravel (7.6 cm) was then placed on top of the packing to stabilize the soil. Sampling ports were fitted with a 0.64-cm ball valve (Swagelok, Niagara Falls, ON, Canada) centrally placed at the bottom of the column to drain the effluent. Air inlets were placed approximately 1.9 cm from the bottom of the column and fitted with a 0.95-cm quick-connect valve (Swagelok, Niagara Falls, ON, Canada).

Aqueous medium (50 mL) was added daily to the top of the column and allowed to infiltrate into the soils. Effluent was collected from the bottom of the columns as it eluted. The nominal hydraulic retention time (based on the infiltration rate and soil field capacity) was approximately 5 d. All column studies were operated with the water content of the soil at field capacity (0.15 g of water/g of soil) and under aerobic conditions. Humidified air was delivered to the column base with a peristaltic pump and allowed to exit to the atmosphere at the top.

Medium compositions were changed periodically in the column experiments but can be grouped into three categories. First was distilled, deionized (DI) water with bicarbonate buffer. Second was tap water with bicarbonate buffer and polyphosphate. Last was a mineral medium containing phosphate buffer and in some cases additional buffering capacity as bicarbonate. Mineral medium was prepared in DI water with the following: MgSO<sub>4</sub>·7H<sub>2</sub>O (50 mg/L), FeSO<sub>4</sub>·7H<sub>2</sub>O (3 mg/L), CaCl<sub>2</sub>·7H<sub>2</sub>O (100 mg/L), NaCl (500 mg/L), H<sub>3</sub>BO<sub>3</sub> (100 mg/L), CaSO<sub>4</sub>·5H<sub>2</sub>O (50 mg/L), ZnSO<sub>4</sub>·7H<sub>2</sub>O (50 mg/L), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (50 mg/L) (11), and variable buffer strength. Table 2 lists, in chronological order, the media composition used in all column experiments.

An initial column study was conducted to determine whether it was possible to stimulate aerobic DNT degradation through the daily addition of medium (50 mL) and air (532 mL at 26.6 mL/min for 20 min). In this 175-d study, duplicate columns served as the experimental systems while duplicate abiotic control columns were operated with sodium azide added to the medium. Subsequent column tests involved three sets of columns, operated in duplicate. The three sets of columns were employed to assess different strategies to sustain and enhance DNT bioremediation of the BAAP soil. The first of the three column sets served as a baseline (repeating the operation of initial experimental systems), being fed mineral medium (50 mL) and air (532 mL at 26.6 mL/min for 20 min) on a daily basis. The second set of columns (enhanced aeration) was operated similarly, except that air was added continuously (24 h/d at 27 mL/min). The third set of columns was operated in a recycle mode. The recycle columns were aerated as carried out in the baseline columns (532 mL at 26.6 mL/min for 20 min), but the composition of the medium added was primarily that of the effluent from the previous day. Of the roughly 50 mL of effluent taken each day, 5 mL was retained for various analyses; the rest (~45 mL) was stored at 4 °C and reused the following day as the influent. Before the stored liquid was added to the column influent, the pH was adjusted to 8.3 with NaOH, and fresh medium was added (~5 mL) to bring the final volume to 50 mL. The three-column system was operated continuously for 90 d.

TABLE 2. Soil Column Studies: Influent Composition Time Line

column <sup>a</sup>	Julian days	medium composition
A, B1	0–45	DI water 10 mM buffer (NaHCO <sub>3</sub> ) pH 7.5
	46–56	tap water 20 mM buffer (Na <sub>5</sub> P <sub>3</sub> O <sub>10</sub> and NaHCO <sub>3</sub> , 1:1) pH 7.5
	57–69	tap water 15 mM buffer (Na <sub>5</sub> P <sub>3</sub> O <sub>10</sub> and NaHCO <sub>3</sub> , 2:1) pH 7.5
	70–85	mineral medium 10 mM buffer (K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> , 1:1) pH 8.3
	86–138	mineral medium 20 mM buffer (K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> , 1:1) pH 8.3
B2, O, R	139–171	mineral medium 40 mM buffer (K <sub>2</sub> HPO <sub>4</sub> , KH <sub>2</sub> PO <sub>4</sub> , and NaHCO <sub>3</sub> , 1:1:2) pH 8.3
	0–4	mineral medium 10 mM buffer (K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> , 1:1) pH 8.3
	5–61	mineral medium 20 mM buffer (K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> , 1:1) pH 8.3
	61–94	mineral medium 40 mM buffer (K <sub>2</sub> HPO <sub>4</sub> , KH <sub>2</sub> PO <sub>4</sub> , and NaHCO <sub>3</sub> 1:1:2) pH 8.3

<sup>a</sup> Column id: A, abiotic control; B1, baseline 1; B2, baseline 2; O, enhanced aeration; R, recycle.

Biodegradation in the columns was evaluated by the use of several indicators. First was the depletion of effluent DNT concentrations in comparison to abiotic controls. Second, nitrite production was quantified. Last, shake flask studies

TABLE 3. Respirometer Studies: Design Matrix

parameter	range tested
baseline	
carbon, nitrogen source	10 mM 2,4-DNT as TOC
medium	mineral medium (100 mL per flask)
buffer	20 mM (10 mM K <sub>2</sub> HPO <sub>4</sub> , 10 mM KH <sub>2</sub> PO <sub>4</sub> )
pH	8.0, maintained with addition of dilute NaOH
bacteria	2,4-DNT degrading culture, enriched from column effluent (approximately 80 mg of washed biomass added in 1 mL of medium)
pH	baseline conditions with initial pH ranging from 5.5 to 9.0 at an increment of 0.5; pH was not maintained over the course of the study
media	baseline conditions with medium strength at 1×, 2×, and 4×
nitrite	baseline conditions with initial nitrite concentrations of 0, 10, 15, 20, and 40 mM
buffer strength	baseline conditions (20 mM phosphate buffer) with additional buffer strength of 0, 20, 40, 60, and 80 mM NaHCO <sub>3</sub> ; pH was not maintained over the course of the study
phosphorus	baseline conditions with phosphate (1:1 K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> ) concentrations varied at 0, 0.4, 0.8, 1.6, and 8.0 mM; initial concentration of 2,4-DNT was 5 mM

were established with column effluent and monitored daily for DNT disappearance and nitrite production. Shake flask studies were conducted using only column effluent (~40 mL) directly transferred to serum vials (120 mL), stirred continuously, fitted with foam stoppers to prevent evaporation, and analyzed for DNT and corresponding end products for a period of 9–10 d. The pH was adjusted to 8.0 with NaOH as needed.

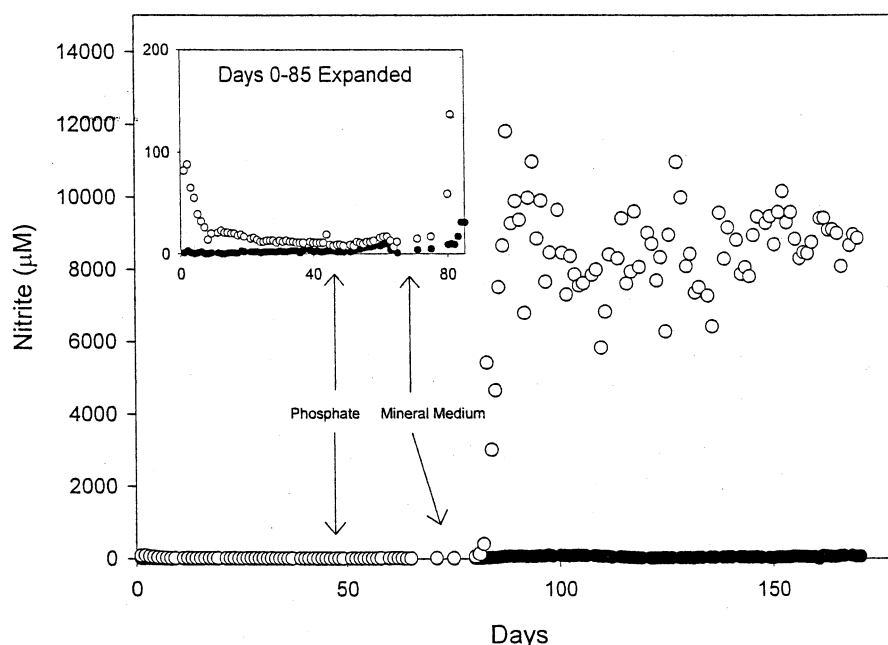


FIGURE 1. Initial soil column studies. Average effluent nitrite concentrations: baseline 1 (○) and abiotic control (●).

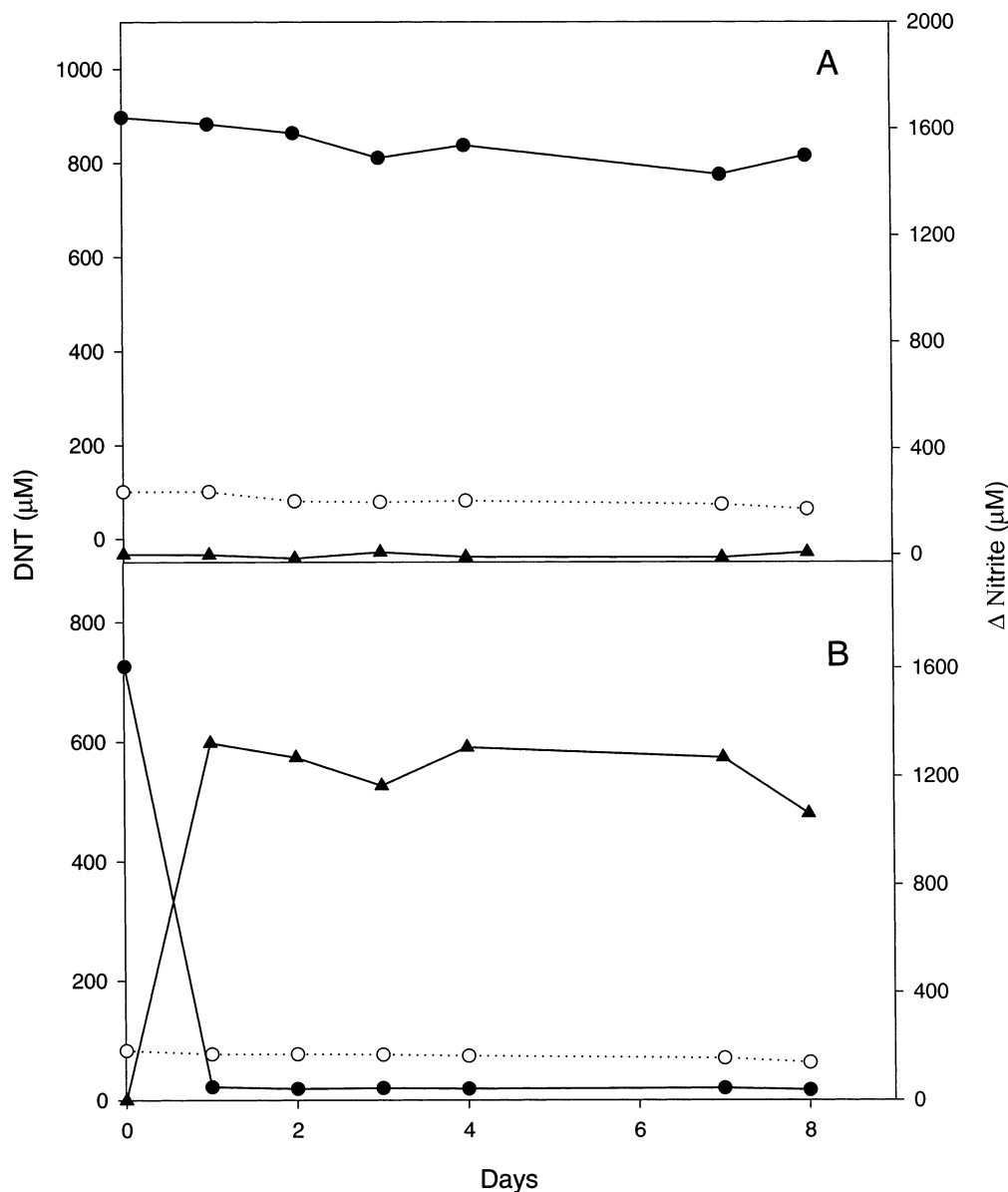


FIGURE 2. Column effluent shake flask studies. (A) Results (averaged) from abiotic control effluent. (B) Results (averaged) from biologically active column (baseline 1) effluent. For both studies, (●) 2,4-DNT concentration, (○) 2,6-DNT concentration, and (▲) as change in nitrite concentration.

To ensure accurate quantification of nitrite, shake flask studies were conducted to determine if biological nitrification, oxidizing nitrite to nitrate, would occur with the nitrite evolved from DNT biodegradation as shown in previous studies (5). The procedure was identical to that described above. Nitrite and nitrate analyses were performed over the course of 10 d.

With the exception of abiotic controls, after all columns had exhibited approximately 90 d of active 2,4-DNT degradation, all systems were destructively sampled by continuous coring through the soil. A zero contamination soil sampler (2.06 cm diameter; Cole-Palmer P-99025-40) was manually driven through the soil, and the resulting soil cores were extruded into plastic sleeves. The cores were then frozen ( $-4^{\circ}\text{C}$ ) and cut into quarters after any gravel from column packing was discarded. These subsamples were analyzed for pore water nitrite levels.

**DNT Enrichment Culture.** A DNT-degrading mixed culture was developed from the original biotic column effluent. As inoculum, approximately 50 mL of effluent was transferred into a flask (2 L) containing mineral medium (900 mL) with

20 mM phosphate buffer and DNT as the only carbon and nitrogen source at concentrations roughly equal to those in column effluents (1000  $\mu\text{M}$  2,4-DNT and 400  $\mu\text{M}$  2,6-DNT). The culture was stirred continuously at room temperature, and nitrite and pH were measured daily. The pH was adjusted each day to 7.5–8.0. When the release of nitrite from the biodegradation of DNT neared 70–80% of theoretical, 10% of the suspension was transferred to fresh medium.

**Respirometer Studies.** 2,4-DNT biodegradation experiments were conducted with the DNT-enrichment culture in a 10-chamber  $\text{O}_2/\text{CO}_2$  Micro-Oxymax respirometer (Columbus Instruments Corp., Columbus, OH) at room temperature. As previously described, both oxygen uptake (5, 10–12) and carbon dioxide production (3, 22) were used to measure DNT biodegradation rates. A confounding factor in some instances where rapid DNT biodegradation took place was the upper quantification limit for  $\text{CO}_2$  evolution by the detector (i.e.,  $\text{CO}_2$  levels exceeded the dynamic range) and  $\text{CO}_2$  production rates were underestimated in those instances. No such difficulty was encountered during  $\text{O}_2$  uptake measurements. In all respirometer experiments, 2,4-DNT comprised the only

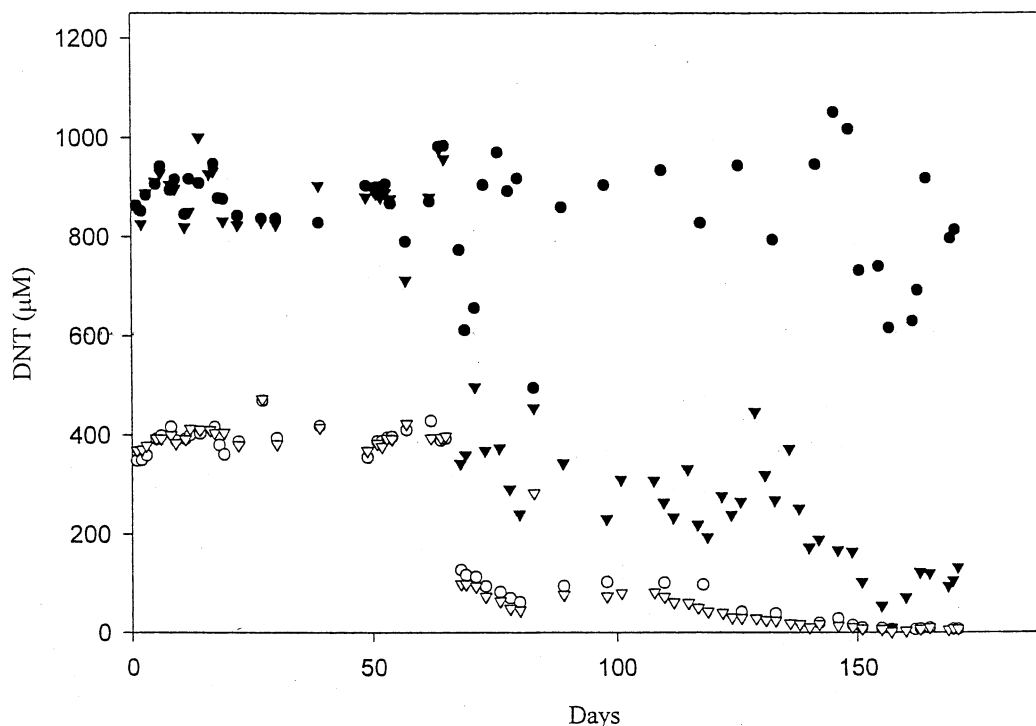


FIGURE 3. Initial soil column studies. Average effluent DNT concentrations: Baseline 1, (▼) 2,4-DNT and (▽) 2,6-DNT. Abiotic control, (●) 2,4-DNT and (○) 2,6-DNT.

organic carbon and nitrogen source. For each experiment, inoculum was taken from the enrichment culture described above. Preparation of the inoculum involved aliquots of the enrichment culture centrifuged (4 °C, 10000g for 10 min) with the supernatant being carefully poured off leaving only the bacterial pellet. The pellet was then resuspended (through vigorous shaking) in DNT-free mineral medium and centrifuged again. This process was repeated for a total of three washes with DNT-free media. In addition, a minimal volume (1 mL) of suspended inoculum was used for each respirometer flask containing 100 mL of mineral medium. Respirometer experiments evaluated the effects of pH, medium concentrations, initial nitrite concentration, phosphorus concentration, and buffer strength on the rate and extent of 2,4-DNT degradation. No carbon dioxide production or oxygen consumption was seen in a respirometer control containing mineral media, 2,4-DNT, but no bacterial inoculum. Table 3 describes the experimental protocols and details the matrix design of varied parameters.

## Results and Discussion

**Column Studies.** Preliminary column studies were conducted to evaluate the stimulation of DNT biodegradation by medium addition and aeration. Nitrite production was used as an indicator of DNT biodegradation (3, 5, 10, 11, 23) (Figure 1). The initial medium was bicarbonate buffer. Over the first 10 d of operation, there was an elevated but decreasing level of nitrite in the effluent from experimental systems. After 20 d of operation, however, nitrite levels in the biotic columns were similar to those in the control columns. On the basis of phosphate limitations cited in previous studies (15, 16, 18), the feed medium was changed on day 45 substituting phosphate for carbonate as a buffer and also as a nutrient. There was no significant increase in nitrite production.

On day 70, the influent was changed to a complete mineral medium, which resulted in the immediate elevation of nitrite levels in the column effluents. After stabilizing, nitrite concentrations averaged 8500  $\mu\text{M}$  ( $\pm 1460$ ) for the remainder of the study. Commensurate with the onset of nitrite

production, the pH of effluent began to decrease. After nitrite levels had stabilized, the pH of effluent medium was typically 6.75–7.25. Appreciable nitrate production was not observed during the study.

As nitrite concentrations stabilized, it was necessary to confirm that nitrite production was an accurate indicator of the DNT degradation activity in the column systems. Therefore, shake flask studies using only column effluent were initiated as described above. Results of these studies (Figure 2) show that 2,4-DNT was immediately depleted within 24 h to levels below the detection limit with a corresponding release of nitrite (approximately 1.7 mol of nitrite released/mol of DNT). Under no circumstances was 2,6-DNT depleted. Identical shake flask studies were conducted periodically as described throughout the remainder of the experimental period with similar results (data not shown). Also, no nitrification (conversion of nitrite to nitrate) was ever observed in these studies.

The effluent 2,4-DNT concentrations in experimental and control systems were similar before the addition of the mineral medium (Figure 3). As concentrations of nitrite began to increase in the active systems, effluent 2,4-DNT levels dropped. No such drop occurred in the controls. Effluent 2,6-DNT concentrations also dropped after approximately 60 d of operation, but the decrease in concentration could not be due to biological activity because similar decreases were observed in both active and control systems. In accordance, periodic effluent shake flask studies showed no loss of 2,6-DNT.

Subsequent column studies were conducted to test whether high levels of 2,4-DNT biodegradation could be induced and sustained by the use of a mineral medium throughout the experimental period. We also sought to evaluate how changes in column operation might influence the extent of degradation achieved. 2,4-DNT degradation began immediately in all systems (Figure 4). Nitrite concentrations increased steadily for approximately the first 20 d and then stabilized. Nitrite concentrations were slightly higher in columns that were continuously aerated or operated

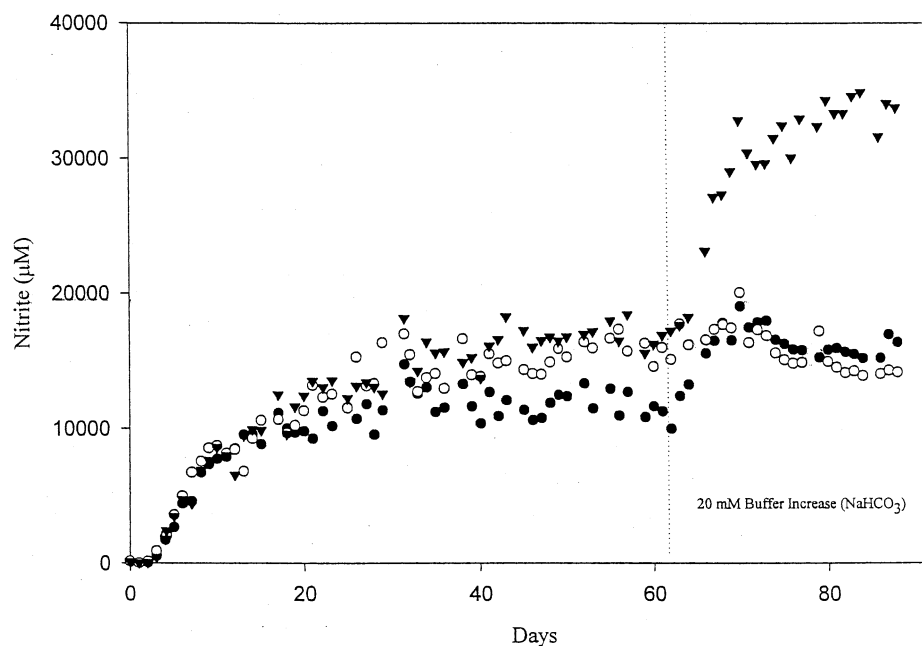


FIGURE 4. Column studies investigating parameter design. Average effluent nitrite concentrations: baseline 2 (●), extended aeration (○), and recycle (▼).

in recycle. Interestingly, the lack of continued nitrite accumulation in the recycle columns indicates a loss of rapid DNT biodegradation after an initial period of biological activity. Shake flask studies conducted, as described previously, confirmed 2,4-DNT biodegradation with no 2,6-DNT biodegradation observed.

The pH in all active columns dropped from 8.3 in the influent to approximately 6.75–7.25 in the effluent due to nitrite production. After bicarbonate buffer was added to the medium (day 61), the average nitrite concentration increased slightly in the baseline columns. In the recycle columns a brief period of increased nitrite production was followed by a decline to a low level.

At the completion of the column studies, soil cores were analyzed for pore water nitrite concentrations to better understand how 2,4-DNT degradation activity was distributed throughout the length of columns. In columns where active 2,4-DNT degradation was occurring, nitrite concentrations increased with the increase in column depth. These results indicate that there was activity throughout the length of the column and suggest that if columns had a longer residence time, additional activity could be expected. Conversely, in abiotic controls and recycle systems, nitrite concentrations remained nearly constant throughout the length of the columns (Table 4). It is important to note that high concentration of nitrite in the recycle columns (Table 4) does not imply continued activity of DNT biodegradation. The pore water nitrite concentration in the recycle columns approached the sum of the total nitrite production throughout the duration of study. As indicated earlier in this paper, 90% of the column effluent in the recycle mode was retained and reused in the following day as the influent diluted only by the 10% addition (vol/vol) of fresh mineral medium.

Estimates of DNT mass removal from the column systems were conducted as described by Ortega-Calvo et al. (23) using effluent DNT concentrations and nitrite analysis. To estimate the washout of both 2,4-DNT and 2,6-DNT, effluent concentrations were summed over the experimental period to provide the cumulative mass of washout that took place (when a sample was not taken on a given day, the concentration was extrapolated using a central differencing method). Similarly, nitrite concentrations were used to calculate the

TABLE 4. Terminal Soil Column Dissections: Pore Water Nitrite Concentrations<sup>a</sup>

column	section	nitrite (pore water) (µM)
abiotic control <sup>b</sup>	1	0.27
	2	0.34
	3	0.34
	4	0.6
baseline 1 <sup>b</sup>	1	179
	2	763
	3	819
	4	2 440
baseline 2 <sup>c</sup>	1	320
	2	1 760
	3	4 260
	4	8 250
enhanced aeration <sup>c</sup>	1	530
	2	1 630
	3	3 340
	4	5 600
recycle <sup>c</sup>	1	39 900
	2	40 800
	3	36 300
	4	34 900

<sup>a</sup> Sections taken in 3.8-cm increments. Spatial distribution is shown as sections 1–4, with section 1 representing the top of the column.

<sup>b</sup> Cores taken after 175 d of operation with the last 90 d being fed mineral media.

<sup>c</sup> Cores taken after 90 d of operation, being fed mineral media for the duration of the study.

2,4-DNT mass that had been degraded based on the stoichiometry presented above where the degradation of 1 mol of DNT yields 1.63 mol of nitrite. As can be seen from Table 5, the measured 2,6-DNT washout was high for all columns other than those operated in recycle, as 90% 2,6-DNT in the effluent from those columns was reintroduced as influent. The percentage of 2,4-DNT that washed out was much less than that of 2,6-DNT due to the higher initial concentrations of 2,4-DNT. Biodegradation (22–30%) of initial 2,4-DNT mass (Table 5) indicates that the biologically active systems have the potential to rapidly reduce the mass of 2,4-DNT present in a vadose zone bioremediation system. In the columns fed mineral medium without recycle, 2,4-

TABLE 5. Average Column DNT Mass Loss as a Percentage of Initial Concentration<sup>a</sup>

column	% washout		% biodegradation		total (% washout + % biodegradation)	
	2,4-DNT	2,6-DNT	2,4-DNT	2,6-DNT <sup>b</sup>	2,4-DNT	2,6-DNT
abiotic control	5.7	81	0.11		5.8	81
baseline 1	3.4	82	19		22	82
baseline 2	1.8	91	25		27	91
enhanced aeration	1.9	85	28		30	85
recycle	0.21	13	5.1		5.3	13

<sup>a</sup> Numbers are the average of the duplicate columns. <sup>b</sup> 2,6-DNT was not found to degrade under any circumstances, thus the only mass was from washout.

DNT depletion from biodegradation was approximately 10 times that of dissolution and washout.

Several common conclusions can be drawn from the comparison of the two sets of column studies. First, the soils were quickly depleted of a required nutrient(s) because phosphate addition alone was not sufficient to support activity. In previous studies investigating rapid DNT biodegradation, 2,4-DNT biodegradation rates were clearly subject to phosphate limitations (16, 18). Similar depletion of phosphate or other nutrient may have occurred in columns after activity was established, but it was not the basis of limited activity prior to the addition of the mineral medium. High rates of 2,4-DNT biodegradation were initiated by providing moisture and mineral nutrients. After the onset of activity, the intermittent addition of mineral medium then became critical to sustain activity in the columns as nutrient concentrations were maintained and nitrite was flushed from interstitial pore water.

A second conclusion from these studies was that 2,6-DNT was not readily biodegraded. Whereas concentrations of both isomers decreased in the effluents of columns, the decrease of 2,4-DNT in the column effluent was likely the result of both the depletion of 2,4-DNT in the soil and the continued degradation in the column drainage system (i.e., gravel and sample reservoir). Shake flask studies with effluent samples revealed a continued rapid degradation of 2,4-DNT, which is consistent with the above hypothesis. Decreases in 2,6-DNT effluent concentrations could not be explained similarly. The fact that effluent shake flask studies did not demonstrate 2,6-DNT degradation activity suggests that the

only loss of 2,6-DNT was through dissolution and washout. In the recycle columns, 2,6-DNT concentrations remained high because the removal of effluent was only 10% of that in other systems. Similar observations regarding the inability to actively stimulate 2,6-DNT degrading activity in BAAP soil has been reported by Nishino and Spain (18).

A third conclusion from these studies (enhanced aeration, Table 5) is that oxygen availability did not strongly affect the level of biodegradation achieved with the water content at field capacity and with periodic aeration. High levels of nitrite production were observed in all systems, and the continuous aeration of columns led to only small increases in activity.

It could not be established from the studies what factor(s) control 2,4-DNT biodegradation after the onset of activity. In systems not operated in recycle, a longer residence time should result in additional production of nitrite that may result in either direct inhibition or cause a pH drop, which can also become inhibitory. If not inhibited by nitrite accumulation, the development of nutrient limitations would be expected to develop as has been observed in ex-situ systems (15). In recycle columns, with long effective contact times, either the slow replenishment of medium, the high nitrite concentrations, or both appear to have caused the inability to sustain degradation activity. Because of the limited size of the column systems, such effects could not be assessed directly, thus a series of batch assays were conducted to evaluate how these various factors would eventually affect the extent of degradation with longer contact times.

**Batch Respirometer Studies.** The first set of batch respirometer studies investigated the direct effect of nitrite

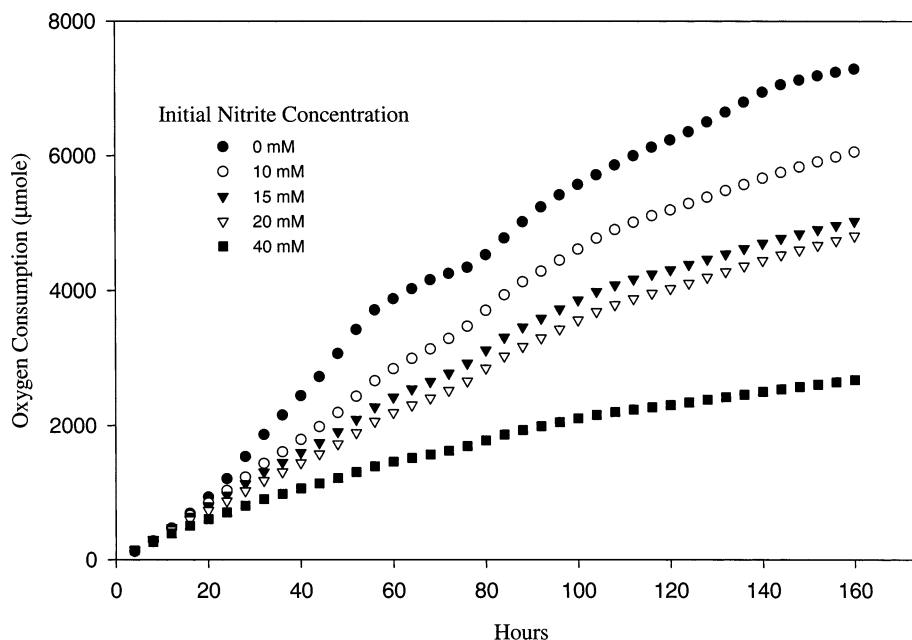


FIGURE 5. Effect of initial nitrite concentration on 2,4-DNT mineralization as measured by oxygen consumption (5.6 mol of O<sub>2</sub> consumed during the mineralization of 1 mol of 2,4-DNT (16)).

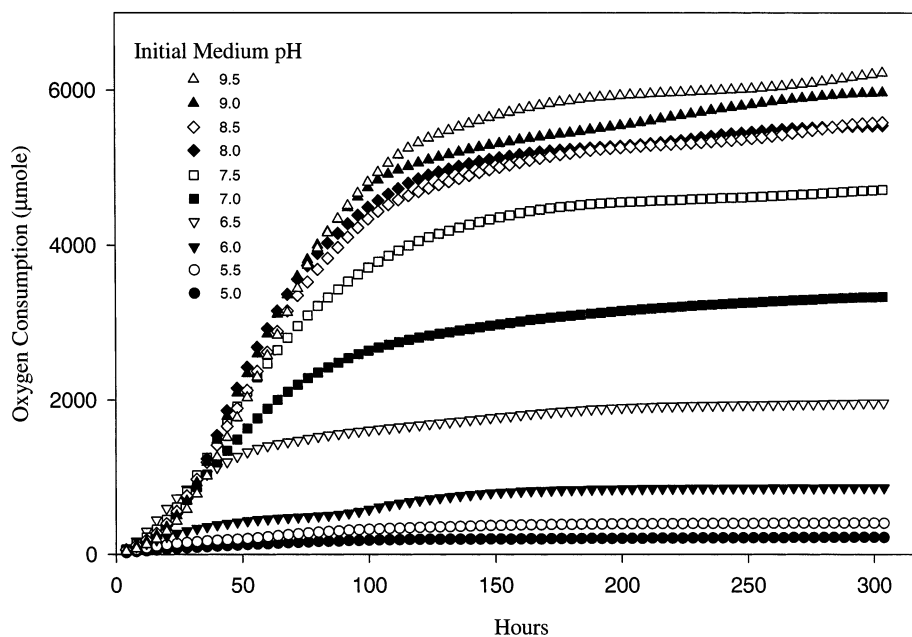


FIGURE 6. Effect of initial pH on 2,4-DNT mineralization as measured by oxygen consumption (5.6 mol of  $O_2$  consumed during the mineralization of 1 mol of 2,4-DNT (16)).

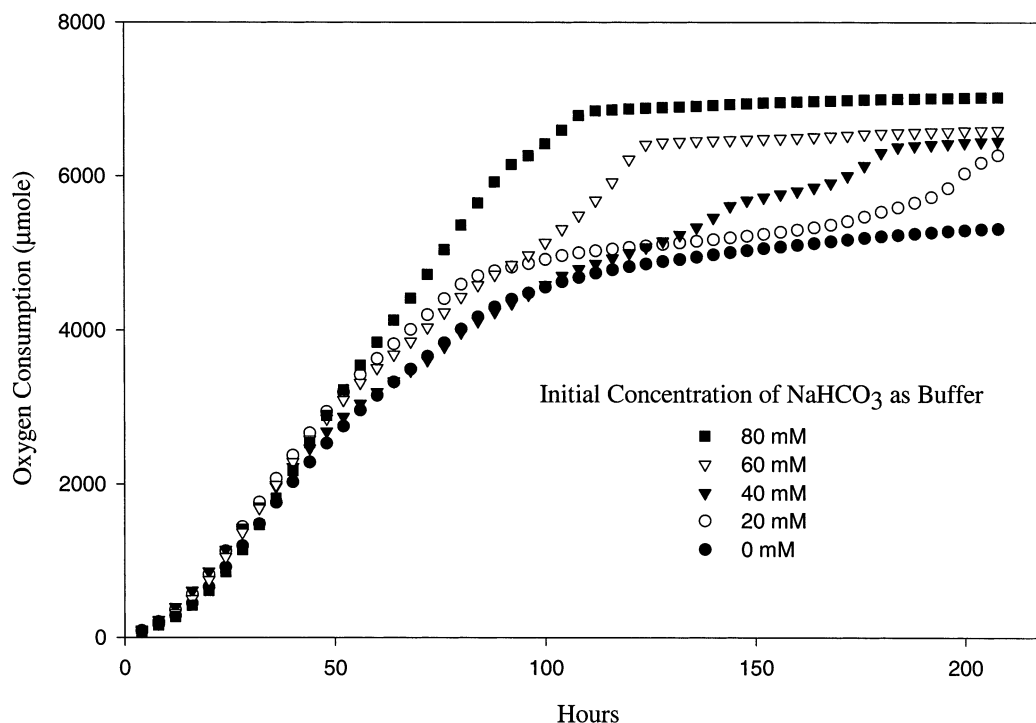


FIGURE 7. Effect of buffering strength on 2,4-DNT mineralization as measured by oxygen consumption (5.6 mol of  $O_2$  consumed during the mineralization of 1 mol of 2,4-DNT (16)).

accumulation due to 2,4-DNT biodegradation and the indirect effect of pH drop from nitrite release. The impact of nitrite accumulation was measured using oxygen uptake and production of carbon dioxide from the aerobic biodegradation of 2,4-DNT at various initial nitrite levels but with the initial pH of 8.5. Increasing nitrite concentrations clearly exert a negative effect on biodegradation rate and extent, with a severe inhibition observed at 40 mM (Figure 5).

A secondary effect of nitrite production is the reduction of pH in the medium. To investigate the impact of pH, respirometer studies were conducted at a range of initial pH values with no nitrite added to the medium. The results (Figure 6) indicate that pH values of 6.0 and below severely

limit the biodegradation of 2,4-DNT. Media with initial pH values of 6.0 and 6.5 supported immediate rapid initial rates of biodegradation that decreased sharply as the pH fell to inhibitory levels. When the initial pH was 7.5 and above, DNT biodegradation was rapid and sustained. Similar observations made by Nishino and Spain strongly support the apparent effect of pH on DNT biodegradation rates (18). Based on the nitrite production and pH drop in column studies, it appears that conditions in the lower portions of the columns were approaching those where reduced rates of 2,4-DNT degradation would be expected. In the case of columns operated in recycle, the accumulation of nitrite probably caused cessation of activity. In columns operated

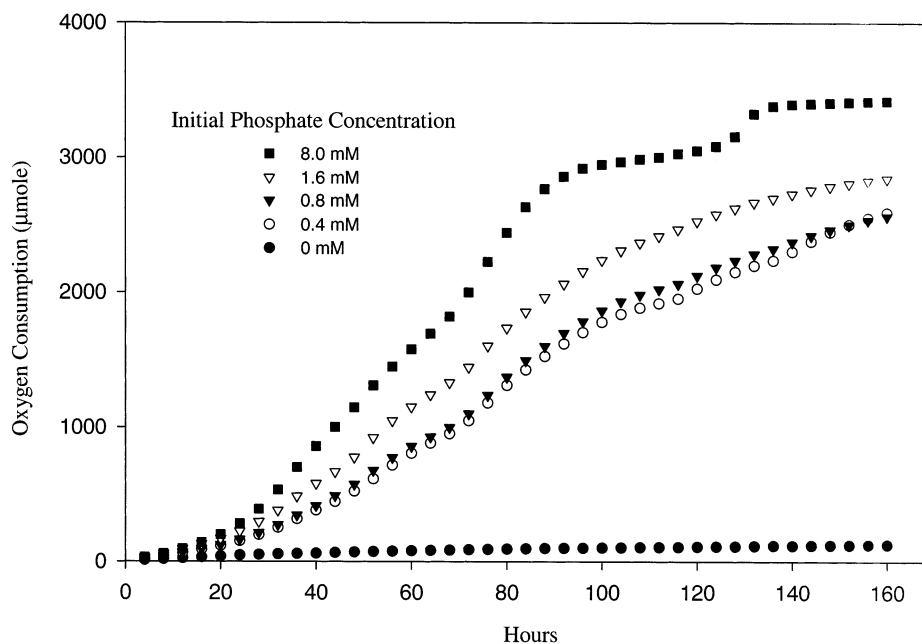


FIGURE 8. Effect of initial phosphate concentration on 2,4-DNT mineralization as measured by oxygen consumption (5.6 mol of  $O_2$  consumed during the mineralization of 1 mol of 2,4-DNT (16)). Note that the initial concentration of 2,4-DNT is 5 mM; normally it is 10 mM 2,4-DNT for baseline conditions.

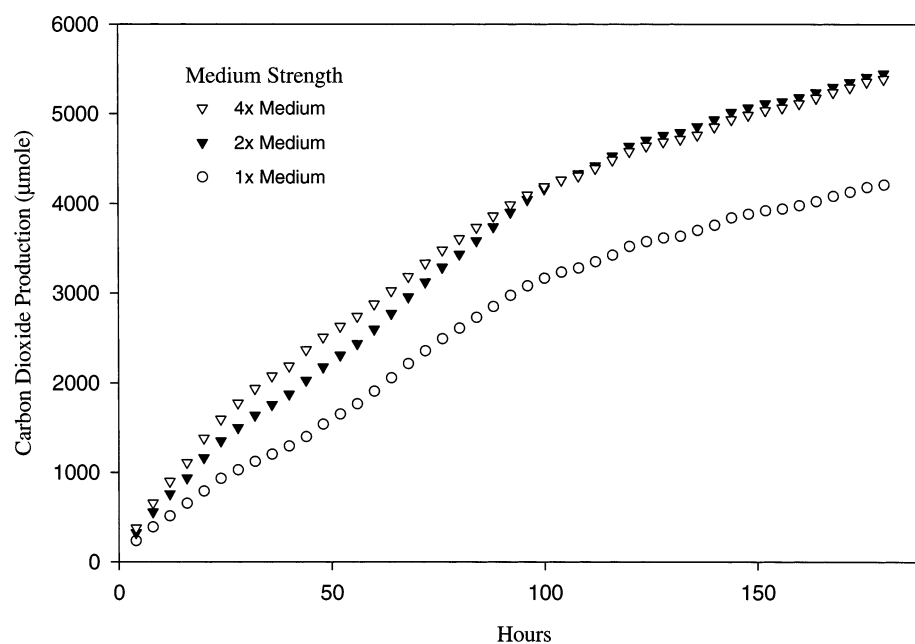


FIGURE 9. Effect of medium strength on 2,4-DNT mineralization as measured by carbon dioxide production (5.2 mol of  $CO_2$  produced during the mineralization of 1 mol of 2,4-DNT (16)).

without recycle, charge balance calculations indicate that accumulation of 15 mM nitrite would have caused the pH drop to the range of 6.0–6.5 assuming 20 mM phosphate buffer and an initial pH of 8.5. At that pH and nitrite concentration, both variables would be expected to reduce rates of biodegradation with pH effects being the most acute. It is of note that the biological oxidation of nitrite to nitrate through nitrification did not occur in columns or effluent shake flask studies. Thus, maintaining high rates of biodegradation will, in part, be controlled by the ability to dilute nitrite concentrations at field capacity (i.e., flushing evolved nitrite from pore water).

A further study investigated the role of the buffering capacity of the medium in maintaining pH and minimizing inhibitory effects. The mineral medium used in column

studies contained 20 mM phosphate buffer. Additional buffer was added in the form of sodium bicarbonate ( $NaHCO_3$ ) at concentrations of 20, 40, 60, and 80 mM. The initial pH was 8.5 with no further adjustment. At all buffer concentrations, 2,4-DNT was degraded immediately (Figure 7). Respiration rates were sustained in accordance with buffer concentration. These results indicate that by increasing buffer capacity of the medium and maintaining a favorable pH range, it is possible to sustain rapid rates of biodegradation for longer periods of time.

In column studies it became apparent that nutrient availability was also an important controlling factor in 2,4-DNT biodegradation activity. Previous studies revealed phosphorus as a limiting nutrient in the biodegradation of DNT in soil slurries (4, 16). To further investigate the limiting effects

of phosphorus, respirometer experiments were conducted using mineral medium with various initial phosphorus concentrations. It is clear that a phosphorus-free medium cannot support the biodegradation process, whereas all phosphorus-amended media supported rapid biodegradation (Figure 8). Media with phosphorus concentrations of 0.4 and 0.8 mM displayed slower biodegradation rates relative to media amended with 1.6 and 8.0 mM phosphorus. Since the phosphate added is also a pH buffer, it is difficult to distinguish between the dual roles of phosphate in these experiments, but it is clear that phosphorus is required at minimal levels as a nutrient to support biodegradation.

On the basis of the results of column studies, nutrient additions are essential to initiate the biodegradation of 2,4-DNT in BAAP soils, and phosphate alone was not sufficient to induce activity. In respirometer experiments, it became apparent that phosphate is critical as a nutrient to induce and sustain degradation. The potential for phosphorus to become a limiting nutrient would be most likely to occur in the recycle mode of operation where the retention time is considerably longer. Interestingly, the analysis of effluent samples taken from the recycle column showed very low effluent phosphate levels (data not shown) as compared to other columns. This suggests that nutrient availability may have contributed to the cessation of activity in the recycle column along with nitrite accumulation and decreasing buffering capacity resulting from phosphate consumption.

A final respirometer study was conducted to investigate the potential to increase biodegradation rates as a function of mineral medium strength (i.e., the concentration of salts in the preparation of the medium). The results (Figure 9) indicated that it was necessary to have the medium concentration (with 20 mM phosphate buffer), which was the concentration used in all previous experiments, to support biodegradation. However, higher concentrations (2 $\times$ , 4 $\times$  media concentration, buffer held constant at 20 mM phosphate) did not increase rates of biodegradation.

The results presented in this study suggest that the recalcitrant nature of 2,4-DNT and 2,6-DNT are the result of different factors. Nutrient availability controlled biodegradation processes for 2,4-DNT when pH and nitrite levels were appropriate. The addition of nutrients did not, however, stimulate 2,6-DNT biodegradation. This inability to stimulate the simultaneous degradation of both isomers when present at high concentrations is consistent with previous reports (5, 10, 15, 17) and represents a challenge for the design of remediation systems. Without intervention (i.e., nutrient addition), the dominant process leading to the loss of DNT from soils will be the dissolution into waters percolating through the vadose zone into groundwater systems.

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## Literature Cited

- (1) Popp, J. A.; Leonard, T. B. In *Toxicity of Nitroaromatic Compounds*; Rickert, D. E., Ed.; Hemisphere Publishing Corp: Washington, DC, 1985; p 53.
- (2) Spain, J. C. In *Biodegradation of Nitroaromatic Compounds and Explosives*; Spain, J. C., Hughes, J. B., Knackmuss, H.-J., Eds.; Lewis Publishers: Boca Raton, FL, 2000; pp 1–6.
- (3) Nishino, S. F.; Spain, J. C.; Lenke, H.; Knackmuss, H.-J. *Environ. Sci. Technol.* **1999**, *33*, 1060–1064.
- (4) Zhang, C.; Hughes, J. B.; Nishino, S. F.; Spain, J. C. *Biodegradation of 2,4-Dinitrotoluene and 2,6-Dinitrotoluene in a Pilot-Scale Aerobic Slurry Reactor System*; Final Report to the U.S. Air Force Research Laboratory on Grant F08637-9B-C-6002; Tyndall AFB, FL, 1999.
- (5) Lendenmann, U.; Spain, J. C.; Smets, B. F. *Environ. Sci. Technol.* **1998**, *32*, 82–87.
- (6) Smets, B. F.; Riefler, R. G.; Lendenmann, U.; Spain, J. C. *Biotechnol. Bioeng.* **1999**, *63*, 642–653.
- (7) Heinze, L.; Brosius, M.; Wiesmann, U. *Acta Hydrochim. Hydrobiol.* **1995**, *23*, 254–263.
- (8) Spain, J. C.; Nishino, S. F.; He, Z. In *Bioremediation of Nitroaromatic and Haloaromatic Compounds*; Alleman, C., Leeson, A., Eds.; Battelle Press: Columbus, OH, 1999; pp 7–14.
- (9) Zhang, C.; Daprato, R. C.; Nishino, S. F.; Spain, J. C.; Hughes, J. B. *J. Hazard. Mater.* **2001**, *B87*, 139–154.
- (10) Nishino, S. F.; Paoli, G. C.; Spain, J. C. *Appl. Environ. Microbiol.* **2000**, *66*, 2139–2147.
- (11) Spanggord, R. J.; Spain, J. C.; Nishino, S. F.; Mortelmans, K. E. *Appl. Environ. Microbiol.* **1991**, *57*, 3200–3205.
- (12) Haigler, B. E.; Nishino, S. F.; Spain, J. C. *J. Bacteriol.* **1994**, *176*, 3433–3437.
- (13) Haigler, B. E.; Johnson, G. R.; Suen, W.-C.; Spain, J. C. *J. Bacteriol.* **1999**, *181*, 965–972.
- (14) Johnson, G. R.; Jain, R. K.; Spain, J. C. *J. Bacteriol.* **2002**, *184*, 4219–4232.
- (15) Zhang, C.; Hughes, J. B.; Nishino, S. F.; Spain, J. C. *Environ. Sci. Technol.* **2000**, *34*, 2810–2816.
- (16) Daprato, R. C.; Zhang, C.; Spain, J. C.; Hughes, J. B. *Water Res.* (submitted for publication).
- (17) Nishino, S. F.; Spain, J. C.; He, Z. In *Biodegradation of Nitroaromatic Compounds and Explosives*; Spain, J. C., Hughes, J. B., Knackmuss, H.-J., Eds.; Lewis: Boca Raton, FL, 2000; pp 7–61.
- (18) Nishino, S. F.; Spain, J. C. In *Bioremediation of Energetics, Phenolics, and Polyaromatic Hydrocarbons*; Magar, V. S., Leeson, A., Eds.; Battelle Press: Columbus, OH, 2001; pp 59–66.
- (19) *Standard Methods for the Examination of Water and Wastewater*; Greenberg, A. E., Clesceri, L. S., Eaton, A. D. Eds.; EPS Group, Inc.: Hanover, MD, 1992; pp 4.85–4.87.
- (20) Keeney, D. R.; Nelson, D. W. In *Methods of Soil Analysis Part 2: Chemical and Microbiological Properties*; Page, A. L., Miller, R. H., Kenney, D. R., Eds.; American Society of Agronomy: Madison, WI, 1982; pp 643–693.
- (21) Chow, J. C. *J. Air Waste Manage. Assoc.* **1995**, *45*, 320–382.
- (22) Bradley, P. M.; Chapelle, F. H.; Landmeyer, J. E.; Shumacher, J. G. *Appl. Environ. Microbiol.* **1994**, *60*, 2170–2175.
- (23) Ortega-Calvo, J.-J.; Fesch, C.; Harms, H. *Environ. Sci. Technol.* **1999**, *33*, 3737–3742.

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